Human placental growth hormone is increased in maternal serum at 20 weeks of gestation in pregnancies with large-for-gestational-age babies

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Abstract

To investigate the relationship between maternal serum concentrations of placental growth hormone (GH-V), insulin-like growth factor (IGF)-1 and 2, IGF binding proteins (IGFBP)-1 and 3 and birth weight in appropriate for gestational age (AGA), large for gestational age (LGA) and small for gestational age (SGA) cases in a nested case-control study. Maternal serum samples were selected from the Screening for Pregnancy Endpoints (SCOPE) biobank in Auckland, New Zealand. Serum hormone concentrations were determined by ELISA. We found that maternal serum GH-V concentrations at 20 weeks of gestation in LGA pregnancies were significantly higher than in AGA and SGA pregnancies. Maternal GH-V concentrations were positively correlated to birth weights and customised birth weight centiles, while IGFBP-1 concentrations were inversely related to birth weights and customised birth weight centiles. Our findings suggest that maternal serum GH-V and IGFBP-1 at 20 weeks' gestation is associated with fetal growth.

Introduction

Delivery of infants with an appropriate birth weight for gestational age is goal of obstetric care. However, inappropriate fetal growth, either large for gestational age (LGA) or small for gestational age (SGA), is common and clinically relevant as they are linked with a number of perinatal complications. LGA is associated with higher rates of Caesarean birth, shoulder dystocia, postpartum haemorrhage and neonatal hypoglycaemia (Weissmann-Brenner et al., 2012), while SGA infants are more likely to be stillborn, and develop perinatal asphyxia, hypothermia and abnormal neurologic symptoms (Doctor et al., 2001; Flenady et al., 2011). Furthermore, both LGA and SGA have been reported to increase the risk for developing certain diseases in later life, such as obesity, type 2 diabetes, hypertension, and dyslipidemia (Barker et al., 1990; Eriksson et al., 2001; Gluckman & Hanson 2004; Lithell et al., 1996).

Humans have two versions of growth hormone (GH) proteins encoded by two GH genes: pituitary GH (GH-N; GH1) and placental GH variant (GH-V; GH2) (Barsh et al., 1983; Hirt et al., 1987). The protein sequences of GH-N and GH-V are highly conserved (93% amino acid identity); however, they have distinct expression profiles. GH-N is mainly secreted in a pulsatile fashion from the pituitary, while GH-V is secreted from the placenta tonically during human pregnancy (Eriksson et al., 1989). The pituitary protein, GH-N (22kDa), is the primary form of GH in maternal circulation up to 15 weeks of gestation (Eriksson et al., 1988). During human pregnancy, GH-V is detected in the maternal circulation from as early as week 5, gradually replacing maternally derived GH-N as the dominant circulating form of GH at approximately 20 weeks of gestation (Eriksson et al., 1989). After that, GH-V concentrations increase significantly to reach a peak at approximately 37 weeks of gestation (Chellakooty et al., 2004; Frankenne et al., 1988). Previous studies have demonstrated a positive relationship between maternal GH-V serum concentration and fetal growth (Chellakooty et al., 2004; Handwerger & Freemark 2000; McIntyre et al., 2000; Mirlesse et

al., 1993; Mittal et al., 2007; Pedersen et al., 2010; Sifakis et al., 2012). Consistently, decreased concentrations of circulating GH-V, and reduced placental mRNA expression of this hormone, have been observed in mid to late stage growth restricted pregnancies, compared to normal pregnancies (Koutsaki et al., 2011; McIntyre et al., 2000). However, whether there is an association between maternal concentration of GH-V earlier in pregnancy and infant birth weight at term is not clear. In the present study we therefore aimed to examine the relationship between maternal serum GH-V concentrations at 20 weeks of gestation and infant birth weight. We hypothesised that maternal serum GH-V concentrations were altered in pregnancies with inappropriate growth babies. Maternal serum IGF-1, IGF-2, IGFBP-1 and IGFBP-3 concentrations were also measured to investigate potential relationships between GH-V and other primary components of the IGF-IGFBP system.

Materials and Methods

Ethical approval was obtained from New Zealand Health and Disability Ethics Committees (AKX/02/00/364/AM03), and all women provided written informed consent. Between November 2004 and October 2007, 2,032 nulliparous women with singleton pregnancies were recruited to the Screening for Pregnancy Endpoints (SCOPE) study in Auckland, New Zealand. The inclusion criteria has been described previously (McCowan et al., 2007).

Participants were interviewed and examined by a SCOPE research midwife at 15 and 20 weeks of gestation. At the first visit, detailed clinical and demographic data were collected. Umbilical artery resistance index (RI) and mean uterine artery RI were measured using Doppler ultrasound at 20 weeks. Maternal serum samples were collected at 20 weeks and stored at -80°C for subsequent analyses. Birth weight was recorded using electronic scales at the time of birth.

In this nested case-control study, 50 LGA and 49 SGA cases were selected and matched to 50 controls (appropriate for gestational age; AGA), matched by ethnicity. Customised birth weight centile was calculated, adjusted for mother's height and weight at 15 weeks' visit, ethnicity, sex and weight of baby and gestation at delivery. SGA and LGA were defined as birth weight $<5^{th}$ and $>95^{th}$ customized birth weight centiles, respectively (McCowan et al., 2004).

Materials

Recombinant human GH-V (22 kDa) was purchased from Protein Laboratories Rehovot (Rehovot, Israel) and was reconstituted in 0.4% $NaHCO_3$ pH 9 (Solomon et al., 2006). Recombinant human GH-N (22 kDa) and placental lactogen (PL) were obtained from the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, CA, US). Human GH-V monoclonal antibodies E8 and 7C12 were obtained from Bio-Rad AbD Serotec (NC, US). E8 does not cross react with GH-N or prolactin. 7C12 shows some cross reactivity with GH-N (5%) as per details provided by the manufacturer.

The development of GH-V ELISA

Due to the lack of a sensitive and specific commercially available assay, an in-house sandwich ELISA was developed and validated for the measurement of GH-V in serum. Mouse anti human GH-V antibodies E8 and 7C12 were tested for specificity for GH-N, GH-V and placental lactogen (PL) using an indirect ELISA (Supplementary Figure 1). E8 was used as the capture antibody due to its binding specificity for GH-V (Igout et al., 1993) (Evain-Brion et al., 1994). 7C12 was used as the detection antibody, and biotinylated using a LYNX Rapid Biotin (Type 1) Antibody Conjugation Kit (Bio-Rad AbD Serotec) according to the manufacturer's instructions. Optimal capture and detection antibody concentrations were determined through standard checkerboard titration procedure (Crowther 1995). A checkerboard titration experiment was conducted empirically using starting concentrations of

8 and 16 µg/ml for capture and detection antibodies respectively, with the antigen (GH-V) at a constant concentration of 5 ng/ml. The optimal capture and detection antibody concentrations were 2 and 8 µg/ml, respectively. Horseradish peroxidase conjugated streptavidin (Bio-Rad AbD Serotec) was used at a concentration of 200 ng/ml as no significant differences were seen at higher or lower concentrations. 2% and 5% bovine serum albumin (BSA), 2% and 5% non-fat dry milk, and a commercial blocking buffer (Ultrablock, Bio-Rad AbD Serotec) were tested under identical ELISA conditions. Ultrablock was found to have the lowest signal-to-noise ratio and was used as the blocking buffer and sample diluent in subsequent experiments. Serum samples from a healthy donor, and sample diluent, were spiked with GH-V at a concentration of 0.625 ng/ml and serial dilutions (neat, 1:2, 1:4 and 1:8) from unspiked, spiked and control samples were assayed. Recovery rate was calculated as the following formula: % Recovery = (Spiked sample values – Unspiked sample values) / Expected values *100. The average recovery rate of this assay was 106%. Linearity was determined by calculating the recovery rate of neat, 1:2, 1:4 and 1:8 dilutions in spiked and unspiked samples, and fell within the acceptance range of 80-120%. Parallelism between the GH-V standards and serial dilutions of serum samples indicated that the standard accurately reflects the GH-V content in natural samples (Supplementary Figure 2). Coefficients of variation (CV) of intra-assay and inter-assay were 4.8% and 6.8%, respectively.

The GH-V ELISA procedure

Microtiter plates were coated with antibody E8 diluted in phosphate buffer (0.1 M Sodium Carbonate, pH 9.5) at a concentration of 2 µg/ml by overnight incubation at 4°C. Coated plates were washed three times with wash buffer (PBS-T; 10 mM phosphate buffer pH 7.4, 150 mM NaCl, 0.05% Tween 20). Blocking was achieved by 1 hour incubation at room temperature with Ultrablock. Standards were prepared from GH-V solution with a range from

0.078 to 5.0 ng/ml. Standards and neat serum samples were incubated for 2 hours at room temperature, then washed three times. All serum samples were measured in duplicate. Biotinylated 7C12 (8 µg/ml) was added and incubated for 1 hour. The plates were washed three times and 200 ng/ml horseradish peroxidase conjugated streptavidin was added and incubated for 30 min. The microtiter plates were then washed four times and 3, 3′, 5, 5′-Tetramethylbenzidine (TMB) Substrate Reagent Set (BD Biosciences) was added to the wells producing a visible signal that is correlated with the amount of antigen. Absorbance was read at 450nm and 590 nm within 30 min of stopping the reaction.

Serum analysis

Serum IGF-1, IGF-2, IGFBP-1 and IGFBP-3 were assayed with human-specific enzyme-linked immunosorbent assays (ELISA) as per the manufacturer's instructions (Mediagnost, Germany).

Statistical analysis

Concentrations of GH-V, IGF-2, IGFBP-1, and IGFBP-3 were positively skewed. Outliers were defined as data points more than 1.5x the interquartile range above the upper or below the lower quartile. Data were log-transformed to improve the approximation of normal distribution and linearize relationships. Data are expressed as means ± S.E.M and median unless stated otherwise. Group means were compared with one way ANOVA with post-hoc analysis (Tukey's procedure). Categorical and numerical variables were compared using chi-square or Fisher's exact test. Pearson's coefficient was used to determine correlations between variables, presented as r values. Linear regression analysis was used to explore the relationships between two variables. Multiple regression was used to determine the association of maternal hormone concentrations with birth weight after controlling for known clinical correlates. All analyses were conducted using IBM SPSS Statistics 21. A p-value of <0.05 was accepted as statistically significant.

Results

The demographic and clinical details of the three groups are shown in Table 1. SGA babies were born earlier with smaller placentas. The mean uterine artery RI at 20 weeks was significantly higher in women destined to deliver SGA neonates (mean uterine artery RI, 0.61) than in women with AGA (0.56) ($p < 0.05$) or LGA pregnancies (0.52) ($p < 0.05$). However, there was no significant difference in umbilical artery RI between groups.

Concentrations of maternal hormones

Maternal serum GH-V concentrations varied between individuals. A few samples had absorbance values falling out of the range of the standard curve. The concentration of these samples was extrapolated from the standard curve, as insufficient samples were available for repeat measurements. Subsequently those extrapolated data were identified as outliers and were not included in the group comparisons (Figure 1A and B). Maternal serum GH-V concentrations at 20 weeks of gestation were significantly higher in LGA pregnancies (median concentration, 2.07 ng/ml) compared to AGA (1.72 ng/ml) ($p < 0.05$) or SGA pregnancies (1.65 ng/ml) ($p < 0.05$), however, there was no significant difference between SGA and AGA samples (Figure 1A and B). There were no significant differences in IGF-1, IGF-2, IGFBP1 and IGFBP-3 concentrations between the groups (Figure 1C, D, E and F). Infant gender did not affect the maternal concentrations of GH-V, IGFs and IGFBPs at 20 weeks (data not shown).

Correlation analysis

One of the aims of this study was to determine whether estimations of maternal GH-related parameters were related to birth weights (Table 2). In the correlation analysis there was a weak but significant positive relationship of maternal GH-V concentrations with birth weights ($r = 0.176$, $p = 0.033$) (Figure 2A), birth weights adjusted for gestational age ($r = 0.174$, $p = 0.035$) and customised birth weight centiles ($r = 0.163$, $p = 0.046$) (Table 2), as

well as placental weights (r = 0.233, p = 0.011). The mean uterine artery RI at 20 weeks was negatively associated with birth weight (r = -0.414, p < 0.0001), birth weight adjusted for gestational age (r = -0.402, p < 0.0001) and customised birth weight centile (r = -0.38, p < 0.0001). Maternal IGF-1 concentrations were related to the changes in GH-V (r = 0.343, p < 0.0001) (Figure 2B), and weakly correlated to changes in IGF-2 (r = -0.168, p = 0.042) and the changes in IGFBP-3 (r = 0.187, p = 0.027). There were no correlations between maternal IGF-I, IGF-2, IGFBP-3 and birth weights. However, maternal IGFBP-1 showed a weak positive relationship with mean uterine artery RI (r = 0.258, p = 0.002) and a weak inverse relationship with birth weights (r = -0.257, p = 0.002) (Figure 2C), birth weights adjusted for gestational age (r = -0.253, p = 0.002) and customised birth weight centiles (r = -0.210, p = 0.011) (Table 2).

Multiple regression was used to determine whether any GH related variables were associated with birth weight after controlling for maternal age, ethnicity, socioeconomic status, smoking and drinking habits, maternal BMI, infant sex and gestation at delivery. Maternal GH-V and IGFBP-1 concentrations were significantly associated with birth weights in separate models that controlled for those factors. Using GH-V and IGFBP-1 in combination with each other, or in combination with the IGF-1, IGF-2 or IGFBP-3 did not improve the model.

Discussion

In the present study, we examined maternal GH-V, IGF-1, IGF-2, IGFBP-1 and IGFBP-3 at 20 weeks of gestation in the maternal samples in pregnancies that later resulted in AGA, SGA or LGA births. This approach has allowed determination of the correlation between GH-related hormones and their relationships with birth weight. We found that maternal serum GH-V was increased at 20 weeks in LGA pregnancies, and that GH-V was positively associated with birth weight.

Most of the studies in mid-late pregnancy found a positive association between maternal GH-V and fetal growth. Chellakooty *et al.* observed that the change in GH-V at 24.5–37.5 weeks was positively associated with fetal growth rate and birth weight (Chellakooty et al., 2004). Two studies reported lower GH-V concentrations at approximately 30 weeks in pregnancies complicated by fetal growth restriction (McIntyre et al., 2000; Mirlesse et al., 1993). Women with pre-eclampsia and SGA have been shown to have lower maternal serum concentrations of GH-V than women with pre-eclampsia but without SGA (Mittal et al., 2007).

As mentioned above, GH-V becomes the dominant circulating form of GH after 20 weeks of gestation. Limited studies have investigated associations of GH-V with fetal growth at earlier time points. Pedersen *et al.* observed increased GH-V concentration in women at weeks 11-14 carrying fetuses with high growth rates assessed by sonographic measurements (Pedersen et al., 2010). However, another study found that maternal GH-V at that period was not associated with birth weight in either SGA or normal pregnancies (Sifakis et al., 2012). Studies that showed no relationship may have been limited by issues of sample size and/or the time of sampling. Our study provides further evidence that maternal GH-V concentration at mid-gestation, as early as 20 weeks, has a positive relationship with birth weight. However, both LGA and SGA can either occur following a pathological process or may represent constitutionally big or small infants. The definitions of LGA and SGA cannot distinguish one process to from the other, and those infants in the AGA range presumably healthy. Further, GH-V has been found to be associated with a number of pathological conditions, such as pre-eclampsia (Mittal et al., 2007), gestational diabetes (McIntyre et al., 2000), and Down's syndrome (Baviera et al., 2004). In this study, we found an association of GH-V with birth weight in LGA, AGA and SGA cases. Whether this association reflects the “normal” or pathological changes of birth weight is still unclear.

GH-V is thought to play a key role in maternal adaptation to pregnancy and fetal growth (Newbern & Freemark 2011). Firstly, GH-V promotes the adaptation to pregnancy of blood vessels supplying the placenta (Lacroix et al., 2005), and relaxes the arteries supplying the uterus (Schiessl et al., 2007); the effect of these changes is an increase in blood flow to the fetus. Secondly, GH-V shares similar physiological somatotrophic, lactogenic and lipolytic properties with GH-N (Alsat et al., 1997; Verhaeghe 2008). The growth-promoting effect of GH-V has also been demonstrated *in vivo* in non-pregnant hypophysectomized rats treated with GH-V and transgenic mice (Barbour et al., 2002; MacLeod et al., 1991; Selden et al., 1988). Thirdly, GH-V increases maternal concentrations of other important growth factors, such as IGF-1 (Caufriez et al., 1990; Caufriez et al., 1994). A highly significant correlation between GH-V and IGF-1 was also found in the present study. Moreover, GH-V has been proposed to be a likely candidate to mediate the insulin resistance of pregnancy in transgenic mice (Barbour et al., 2002), where GH-V may impact on maternal metabolism and substrate supply to the fetus, either directly or mediated by IGF-1.

IGF-1 and IGF-2 mediate a range of actions in many tissues including stimulation of cell growth, cell survival and differentiation. These actions are regulated by a series of specific binding proteins, which may inhibit or enhance IGF activity (Baxter 2000). There is clear evidence that the IGFs and IGFBP family are closely related to fetal growth (Boyne et al., 2003; Chard 1994; Han et al., 1996; Hills et al., 1996; Holmes et al., 1997), however, their associations were mostly determined at mid to late pregnancy. In the present study, we found that maternal serum IGFBP-1 as early as 20 weeks was negatively related to birth weight, but IGF-1, IGF-2 and IGFBP-3 had no association with birth weight at that time. Maternal serum IGFBP-1 may therefore be a potential biochemical marker for early detection of inappropriate fetal growth.

IGFBP-1 binds to only a small proportion of circulating IGF-1 (Frystyk et al., 2002). However, it is considered to be important for short-term regulation of IGF bioactivity, since IGFBP-1 concentrations fluctuate in response to insulin and carbohydrate intake (Baxter 1995). Previous studies have demonstrated two major roles of IGFBP-1. IGFBP-1 serves as an endocrine factor to regulate the bioavailability of serum IGF-1, inhibits IGF binding to cell surface receptors, and thereby inhibits IGF-mediated cell mitogenic and metabolic actions. Such a mechanism can be reconciled with observations showing that serum concentrations of 'free' IGF-1 are markedly increased during late pregnancy, consequent on IGFBP-3 proteolysis and decreased ternary complex formation (Skjaerbaek et al., 2004). The IGF inhibitory actions of IGFBP-1 have been confirmed by *in vitro* studies and *in vivo* animal investigations (Jones et al., 1991; Lee et al., 1993; Lowman et al., 1998; Rajkumar et al., 1995). Overexpression and subsequent excess levels of circulating IGFBP-1 result in inhibition of fetal growth and the metabolic effects of the IGFs (Murphy et al., 1995; Rajkumar et al., 1995), while low IGFBP-1 concentrations are associated with macrosomia and insulin resistance syndromes (Heald et al., 2001; Janssen et al., 1998; Yan-Jun et al., 1996). Consistent with previous studies (Harrington et al., 1997; Melchiorre et al., 2009), we found that uterine artery RI at 20 weeks was increased in pregnancies with SGA and was negatively associated with birth weight. In addition, a positive relationship between IGFBP-1 and uterine artery RI was observed. However, there is no robust evidence suggesting a direct vasodilating effect of IGFBP-1, although one study demonstrated a reduction in the maximal vasoconstrictor response in aorta of IGFBP-1-overexpressing mice (Wheatcroft et al., 2003). The other role of IGFBP-1 is to act as an autocrine/paracrine factor in the female reproductive system (Rutanen & Seppala 1992). In humans, IGFBP-1 is synthesised in large amounts by the secretory endometrium, ovarian granulosa cells and decidualized stromal endometrial cells of early pregnancy (Koistinen et al., 1990; Rutanen et al., 1985). IGFBP-1,

317 together with IGFs, ovarian steroids and other factors, are involved in a complex system
318 which regulate decidualization, trophoblast invasion, and fetal growth (Hamilton et al., 1998;
319 Han et al., 1996).
320 In conclusion, maternal serum GH-V and IGFBP-1 concentrations at 20 weeks of gestation
321 were significantly associated with birth weight. However, given the strength of the
322 association, a potential clinical use as early markers for aberrant fetal growth patterns would
323 require further investigation. As an association between maternal circulating GH-V and SGA
324 has been observed later in the third trimester, and measurements taken later in pregnancy may
325 be more informative.
326

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330

331 **Conflict of Interest**
332 The authors declare that they have no conflict of interest.
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Figure Legends

Figure 1. Serum GH-V, IGF-1, IGF-2, IGFBP-1 and IGFBP-3 concentrations.
Data are showed as Tukey box-whisker plots (median, 25th centile, 75th centile and range). Groups which do not share the same letter are significantly different from each other ($p<0.05$). Outliers are presented as hollow symbols.

Figure 2. (A): The association of maternal GH-V concentration and birth weight. (B): The association of GH-V and IGF-1. (C): The association of maternal IGFBP-1 concentration and birth weight.
GH-V and IGFBP-1 concentrations here are log-transformed.